Fig. 5.10a  Protocol for pDNA vaccination with the mutant p53 tumor antigen. 70 µg pDNA were solubilized in PBS or mixed with the liposomal transfection reagent DOTAP and injected intradermally into the ears or food pads of Balb/c mice. Injection was repeated 6 times in 10 days intervals. Two months after onset of immunization mice were inoculated with 5 x 10^6 Meth A tumor cells. Tumor resistant mice were re-challenged after 5, 8, and 12 months with Meth A or Q6 tumor cells.

Fig. 5.10b  Immunization of Balb/c mice with mutant p53 pDNA constructs protects mice against tumor cell growth. Balb/c mice were immunized six times with a vector construct encoding a mutant p53 tumor antigen or a pCMXLacZ-control vector two months before tumor cell inoculation. Injection of pCMX-mtp53 protected mice from Meth A tumor growth (right). Mice immunized with a control construct developed large tumors three weeks post tumor cell inoculation (left).
Fig. 10c Injection of mutant p53 encoding constructs protects mice from tumor cell growth. Balb/c mice were injected intradermally with different pDNA constructs. pDNA was solubilized in PBS or formed liposomal complexes when mixed with the transfection reagent DOTAP. Ears or food pads were injected with the transgene. Two months after onset of immunization Balb/c mice were challenged with $5 \times 10^6$ Meth A tumor cells. All mice injected with pCMX, pCMXLacZ, pZeoSV and pZeoSVLuc developed large tumors after three weeks and eventually died. Also, athymic nude mice were not protected from Meth A tumor growth when injected with the mutant p53 tumor antigen pDNA. However, subcutaneous injection of plasmid vectors encoding mutant p53 generates an effective and protective immune response to Meth A tumor cells. Injection into the ears of mice proved to be most efficient.

### 5.3.3 Minimal requirements for pDNA-vaccination

Rejection of Meth A tumor cells is dependent on the number of pDNA inoculations and the time period to pass before tumor cell transplantation (Fig. 5.11). Mice were injected intradermally with 70 µg pDNA in 10 days intervals. Rejection of Meth A tumor cells was most efficient after 60 days. Short termed immunizations (> 30 days) had no effect on tumor rejection (Fig. 5.11).

For biolistic particle bombardment 1 µg of pDNA was coated to gold particles and used for immunization. A protective immune response and Meth A tumor cell rejection was observed after 60 days and six immunizations (Fig. 5.11). In general, immunization by biolistic gene transfer was less effective than injection of the pDNA vaccine. Biolistic transfer is known to preferentially activate the Th2 subpopulation of T lymphocytes, whereas direct injection of naked DNA was connected to Th1 induction (251). The Th2 subset of lymphocytes is involved in the production of cytokines like IL4, IL5, IL6, and IL10 that promote B cell activation and immunoglobulin class switching. However, type 1 like helper T cells support development of cellular immune responses, including CTL and the IgG1a immunoglobulin subtype by production of cytokines like IL2 and interferon-γ. It has been demonstrated by many groups that the type of T cell help that is elicited is critical to the outcome of the disease. For example, infection by Leishmania is lethal in mouse strains that develop Th2 responses to the infection, whereas strains that develop Th1 responses become immune (252).
V. Results

**Development of protective immunity by pDNA vaccination**

![Graph showing number of protected mice after DNA vaccination.]

**Fig. 5.11** Time required for efficient DNA-vaccination. Balb/c mice were treated with a mutant p53-vector construct. Subcutaneous injection into the ears has been performed in 10 days intervals (70 µg DNA/injection). DNA-vaccination and protection of Balb/c mice from Meth A tumor growth is most efficient after 60 days.

**Immunization of mice by biolistic particle bombardment**

![Graph showing number of protected mice after DNA vaccination.]

**Fig. 5.12** Immunization of Balb/c mice by biolistic particle bombardment. Gold particles were coated with 1 µg pDNA and delivered to ears of mice in 10 days intervals. 5 days after the last immunization 5 x 10^6 tumor cells were transplanted subcutaneously into the flank. Immunization was inefficient after 2 or 4 inoculations. However, after 6 gene gun inoculations 50% of the treated animals were protected from tumor cell growth.
5.3.4 pDNA-vaccination is not effective in mice with established tumors

We wanted to analyze the therapeutic benefit of DNA plasmids as anti-tumor vaccines to reduce established tumors. Meth A tumor cells were transplanted to the flank of Balb/c mice and formed solid tumors. Then, mice with established tumors were treated with pDNA constructs and the therapeutic effect was analyzed by measuring tumor growth (Fig. 5.13). Tumor growth was not influenced by the DNA-vaccine. Previous experiments showed that immune effector mechanisms were effectively activated 2 months after onset of pDNA-vaccination. However, tumors in unprotected mice already kill the animals within 1 month. Our experiments suggest that treatment of mice with established tumors did not allow the formation of an effective immune response in time and mice are killed by the tumor before the immune system can eliminate tumor cells or lead to tumor regression. The activation of immune effector mechanisms might also be inhibited by the production of immunosuppressive factors secreted by the solid tumor. These factors might produce a microenvironment unfavorable for lymphocyte action and other immune modulating cells.
Treatment of mice with DNA encoding a mutated tumor suppressor gene after tumor cell transplantation

Fig. 5.13 DNA treatment of mice with established tumors. Balb/c mice were inoculated with $5 \times 10^6$ Meth-A tumor cells. Tumor growth was monitored with vernier callipers. DNA-vaccination was started at day 2 or day 8 post tumor cell transplantation. 70 µg of pDNA were injected subcutaneously in 5 day intervals. An efficient anti-tumor response could not be achieved.
5.3.5 pDNA-immunization effectively protects mice from the establishment of pulmonary metastases

The treatment of disseminated metastases is still a most difficult task for the medical oncologist. The primary tumor can normally be excised by surgical means, however disseminated metastases are much more difficult to be treated and are the main reason for death in cancer patients. For this reason we investigated the efficacy of the pDNA-vaccine to suppress metastases formation in a mouse model system. Meth A tumor cells form extensively pulmonary metastases upon intravenous transplantation into Balb/c mice. 5 x $10^5$ tumor cells were injected in the lateral tail vein of mice at 6 to 8 weeks of age. Survival of mice and the presence of disseminated tumor foci in different organs were analyzed 5 weeks post tumor cell inoculation (Fig. 5.15). For histopathology liver, spleen, kidney, and lung were excised from mice challenged with tumor cells, fixed in 4% buffered formalin and embedded in paraffin blocks. Tissue specimen were sectioned at 4 µm, stained with eosin/hematoxylin and examined for pathological findings by the Kantonspital Basel, Institut für Pathologie, Basel, Switzerland.

pDNA-vaccinated mice did not display any tumor formation in lung, spleen, kidney, and liver (Fig. 5.14). However, untreated mice showed disseminated tumor nodules in the lung. Tumors were identified as metastases of an undifferentiated solid carcinoma, infiltrating the pleura (Fig. 5.14). Histopathological analysis did not reveal metastasis formation in any other major organ of these mice (data not shown). Additionally the survival of mice post tumor cell inoculation was evaluated. We observed that 100% of the non-treated animals died within 6 weeks, whereas all of the treated animals survived for longer than 12 months (Fig. 5.15). These results show that pDNA vaccination efficiently protects mice from the development of pulmonary metastases. Desection of the primary tumor, followed by pDNA-vaccination against identified tumor antigens might prove as an effective means to protect against reoccurrence of tumors or it might result in a prolonged disease free time period for cancer patients.

![Fig. 5.14 Histopathological analysis of the lungs of mice transplanted with Meth A tumor cells. Sections of lung tissue 35 days after tumor cell inoculation from (A) a Meth A transplanted untreated control animal (multiple, extensive metastases of an undifferentiated carcinoma, marked by an arrow) or (B) a Meth A transplanted mouse, pre-treated with pDNA-vaccines containing the mutant p53 tumor antigen (normal lung, no microscopically detectable tumor cells).](image)
pDNA immunized mice resistant to solid tumor growth are also protected from metastases

Fig. 5.15 Formation of metastases is prevented in DNA-immunized mice. Balb/c mice were intravenously injected with 5 x 10^5 Meth A tumor cells. Untreated control mice were unprotected from metastasis. However, Balb/c mice, immunized by the mutant p53 DNA vaccine, suppressed the formation of pulmonary metastases.

5.1.4 Identification of effector mechanisms

5.1.4.1 The humoral immune response in pDNA vaccination

Administration of pDNA has proven to be an effective means of generating humoral immune responses specific for the transgene. Transgene expression and the duration of antibody responses induced by pDNA vaccination is long lived in mice as shown by different groups (253, 254). The antibody isotypes induced are generally IgG, but serum IgM and IgA also have been detected (254, 255). In mice the subclass of serum antibodies induced by DNA-vaccination is predominantly IgG_{2a} suggesting that the generation of Th1-like T cell help may be a general property of DNA-vaccines. However, vaccination using a gene gun appears to shift immune responses toward Th2-like responses that is typified by a predominance of the IgG_{1} immunoglobulin isotype (256).

To identify effector mechanisms that are responsible for Meth A tumor rejection we tested mouse sera for the presence of mutant p53 specific antibodies. Serum samples from mice were taken from the lateral tail vein 10 days after the last pDNA vaccination and assayed for anti-p53 antibodies using a Western blot procedure. The monoclonal antibody PAb-431 (Dianova, Hamburg, Germany) was used as a positive control. Briefly, EcoR I Hind III fragments of the mutant p53 alleles were cloned into the bacterial expression vector pRSET (Invitrogen, NV Leek, The Netherlands) and the recombinant vector molecules were transformed into competent E. coli BL21-(DE3)LysS. Glycerol stocks were used for inoculation of over night cultures that were diluted in LB medium, 0.6% glucose, 50 µg/ml.